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INTERNATIONAL APPLICATION PUBLISHED

WO 9602642A1

(51) International Patent Classification 6 :	C12N 15/12, C07K 14/82, 14/47, G01N 33/574, A61K 38/17, 38/04	A1	(43) International Publication Date: 1 February 1996 (01.02.96)
(21) International Application Number:	PCT/GB95/01719		
(22) International Filing Date:	20 July 1995 (20.07.95)		
(30) Priority Data:	08/277,660 20 July 1994 (20.07.94) 08/424,957 19 April 1995 (19.04.95)	US	
(71) Applicant (for all designated States except US):	UNIVERSITY OF DUNDEE [GB/GB]; Dundee DD1 4HN (GB).		
(72) Inventors; and			
(75) Inventors/Applicants (for US only):	PICKSLEY, Steven, Michael [GB/GB]; 52 Maule Street, Carnoustie, Angus DD7 6AB (GB). LANE, David, Philip [GB/GB]; Magicwell House, Balmullo, St. Andrews, Fife KY16 0AN (GB).		
(74) Agents:	GREAVES, Carol, P. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		

(54) Title: INTERRUPTION OF BINDING OF MDM2 AND p53 PROTEIN AND THERAPEUTIC APPLICATION THEREOF

(57) Abstract

Compounds for use in interfering with the binding between p53 and MDM2 or a protein having a p53 binding site analogous to that of MDM2, the compound being selected from the group consisting of a peptide having up to twenty-eight amino acids which is able to disrupt or prevent binding between p53 and MDM2, or a functional peptide analogue thereof. Methods for detecting such compounds and their application in the diagnosis and treatment of tumours is also described and claimed.

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INTERRUPTION OF BINDING OF MDM2 AND P53 PROTEIN AND
THERAPEUTIC APPLICATION THEREOF

5 The invention relates to the area of cancer detection and
therapeutics. More particularly it relates to the
prevention or disruption of the inactivation of the p53
tumour suppressor which occurs as a result of the binding
of a protein through the amino acid motif within the
10 region of p53 represented by amino acids 16-30
QETFSDLWKLLPENN (SEQ ID NO:1) of the human p53 protein.
An example of such a protein is the oncogene protein MDM2
(human MDM2).

15

Inactivation of the p53 tumour suppressor is a frequent event in human neoplasia. The inactivation can occur by mutation of the p53 gene or through binding to viral or cellular oncogene proteins, such as the SV40 large T antigen and MDM2. While the mechanism through which wild-type p53 suppresses tumour cell growth is as yet poorly defined it is clear that one key feature of the growth suppression is the property of p53 to act as a transcription factor (Farmer, G., et al. (1992). Nature, 358, 83-86; Funk, W.D. et al. (1992). Mol. Cell. Biol., 12, 2866-2871; Kern, S.E., et al. (1992). Science, 256, 827-830). Currently, considerable effort is being made to identify growth control genes that are regulated by p53 binding to sequence elements near or within these genes. A number of such genes have been identified. In cases such as the muscle creatine kinase gene (Weintraub, H., et al. (1991). Proc. Natl. Acad. Sci. U.S.A., 88, 4570-4571; Zambetti, G.P., et al. (1992). Genes Dev., 6, 1143-1152) and a GLN retroviral element (Zauberan, A., et al. (1993). Embo J., 12, 2799-2808) the role these genes might play in the suppression of growth control is unclear. Yet there are other examples, namely *mdm2*

(Barak, Y., et al. (1993). *Embo J.*, 12, 461-468; Wu, X., et al. (1993). *Genes Dev.*, 7, 1126-1132) GADD 45 (Kastan, M.B., et al. (1992). *Cell*, 71, 587-597) and WAF1 or CIP1 (El-Beiry, W.S., et al. (1993). *Cell*, 75, 817-825; 5 Harper, J.W., et al. (1993). *Cell*, 75, 805-816) where their involvement in the regulation of cell growth is better understood.

In the present text "mdm2" refers to the oncogene and 10 "MDM2" refers to the protein obtained as a result of expression of that gene.

Mdm2, a known oncogene, was originally found on mouse double minute chromosomes (Cahilly-Snyder., L., et al. 15 (1987) *Somatic Cell Mol. Genet.* 13, 235-244). Its protein product was subsequently found to form a complex with p53, which was first observed in a rat fibroblast cell line (Clone 6) previously transfected with a temperature sensitive mouse p53 gene (Michalovitz, D., et al. (1990). *Cell*, 62, 671-680). The rat cell line grew well at 37°C but exhibited a G1 arrest when shifted down to 32°C, which was entirely consistent with an observed 20 temperature dependent switch in p53 conformation and activity. However, the p53-MDM2 complex was only observed in abundance at 32°C, at which temperature p53 25 was predominantly in a functional or "wild-type" form (Barak, Y. et al. (1992). *Embo J.*, 11, 2115-2121 and Oren, 1992; Momand, J., et al. (1992). *Cell*, 69, 1237-1245). By shifting the rat cell line down to 32°C and 30 blocking de novo protein synthesis it was shown that only "wild-type" p53 induced expression of the mdm2 gene, thereby accounting for the differential abundance of the complex in terms of p53 transcriptional activity (Barak, Y., et al. (1993). *Embo J.*, 12, 461-468) 35 The explanation was further developed by the identification of a DNA binding site for wild-type p53 within the first intron of the mdm2 gene (Wu, X., et al.

(1993). Genes Dev., 7, 1126-1132). Reporter constructs employing this p53 DNA binding site revealed that they were inactivated when wild-type p53 was co-expressed with MDM2.

5

This inhibition of the transcriptional activity of p53 may be caused by MDM2 blocking the activation domain of p53 and/ or the DNA binding site. Consequently, it was proposed that *mdm2* expression is autoregulated, via the 10 inhibitory effect of MDM2 protein on the transcriptional activity of wild-type p53. This p53-*mdm2* autoregulatory feedback loop provided a novel insight as to how cell growth might be regulated by p53. Up to a third of human sarcomas are considered to overcome p53-regulated growth 15 control by amplification of the *mdm2* gene (Oliner, J.D., et al. (1992). Nature, 358, 80-83). Hence the interaction between p53 and MDM2 represents a key potential therapeutic target.

20 The cDNA sequence encoding the human MDM2 protein (which is also referred to as "HDM2" in the art) is known from WO93/20238. This application also discloses that human MDM2 protein binds with human p53 and it has been suggested that molecules which inhibit the binding of

25 MDM2 to p53 would be therapeutic by alleviating the sequestration of p53. However it is also suggested that the p53 and MDM2 binding site is extensive, including amino acid residues 13-41 of p53 as well an additional nine to thirteen residues at either the amino or carboxyl 30 terminal side of the peptide are also involved. This would indicate that a large polypeptide or other large molecule would be required in order to significantly interfere with the binding.

35 The applicants have therefore sought to immunochemically characterize the p53-MDM2 complex, and also determine in fine detail the MDM2 binding site on p53.

Surprisingly, it has been found that only a relatively small number of amino acids within the p53 protein are involved in binding to MDM2.

5

The precise identification of this binding site is vital to allow the rational design of molecules which will disrupt or prevent binding between p53 and MDM2 or proteins containing analogous p53 binding sites. In addition it allows for the design of screening procedures which will enable compounds which can disrupt or prevent the binding interaction to be accurately and rapidly identified.

15 The applicants have found that the site on the p53
protein which is responsible for binding to MDM2 is a
small sequence of only six amino acids, of which three
amino acids have been found to be critical. This
sequence is represented by the sequence TFSDLW (SEQ ID
NO:2) in human (amino acids 18-23 in the sequence) and
TFSGLW (SEQ ID NO:4) (amino acids 18-23) in mouse, of
which the critical amino acids appear to be F--LW (SEQ ID
NO:4). By disrupting or preventing p53 from binding in
this specific region, the deleterious effects of binding
to MDM2 or proteins having an analogous p53 binding site
can be avoided. Proteins having a p53 binding site which
is analogous to that of MDM2 will generally comprise
oncogene proteins which bind to p53 through the amino
acid motif within the region of p53 represented by amino
acids 16-30 (QETFSDLWKLLPENN) (SEQ ID NO:1) of the human
p53 protein.

This finding has recently been reinforced by a report that two components of the transcriptional machinery, namely TAFII40 and TAFII60, require Leu-22 and Trp-23 for them to bind to p53 and mediate p53 transcriptional activity (Thut- CJ, et al., 1995, Science 267:100-4).

The same two amino acids of p53 are critical for the binding of MDM2 as disclosed above as well as Ad E1b, thus strengthening the hypothesis that MDM2 and E1b act by binding to p53 and blocking the transcriptional 5 activity of p53.

Hence the present invention provides a method for interfering with the binding between p53 and MDM2 or an oncogene protein having an analogous p53 binding site, 10 which method comprises administering an effective amount of a compound, selected from the group consisting of a peptide having up to twenty eight amino acids which is able to disrupt or prevent the binding between p53 and MDM2, or a functional peptide analogue thereof.

15 It may be expected that small peptides, for example of from 4 to 10 amino acids, suitably from five to 10 amino acids, or peptide analogues thereof would be particularly suitable in such a process. Peptides which would be of 20 particular interest are those which show a consensus with the fragment of p53 which has been found to be crucial for binding. Such peptides include fragments of p53 protein which includes at least some of amino acids 18-23 within the sequence of human p53, as identified in 25 WO93/20238 or a peptide analogue thereof. Suitably these peptides are those which are circular, linear or derivatised to achieve better penetration of membranes.

30 Novel peptides or peptide analogues of this type form a further aspect of the invention.

Hence preferred peptides include the sequence FxxLW (SEQ ID NO:4) such as TFSDLW (SEQ ID NO:2) or a portion thereof. As used herein, 'x' refers to any amino acid. 35 In a preferred embodiment, an aspartate residue in the sequence is replaced by a glutamate residue so that the sequence is FxELW (SEQ ID NO:5 such as TFSELW (SEQ ID

NO:6.

Other compounds which may interfere with the binding include organic compounds which are modelled to achieve
5 the same three dimensional structure as the said region of the p53 peptide. Hence in an alternative embodiment the invention provides an organic compound which is modelled to resemble the three dimensional structure of the amino acids represented by the sequence F--LW (SEQ ID NO:4) as it appears in human p53 in the region of amino acids 19-23 and which binds to human MDM2. In particular the organic compound may be modelled to resemble the three dimensional structure of the sequence TFSDLW (SEQ ID NO:2) as it appears in the region of amino
10 acids 18-23 of human p53.
15

A suitable oncogene protein is MDM2 but the disruption of binding of p53 to other oncogene proteins containing a p53 binding site analogous to that of MDM2 are included
20 within the scope of the present invention. Examples of other such oncogene proteins include the adenovirus E1B 58kD protein, the Tata box binding protein TBP and the transcription factor of the E2F family.

25 As used herein the expression 'peptide analogue' refers to peptide variants or organic compounds having the same functional activity as the peptide in question, in particular which interfere with the binding between p53 and MDM2. Examples of such analogues will include
30 chemical compounds which are modelled to resemble the three dimensional structure of the sequence TFSDLW, (SEQ ID NO:2) and in particular the arrangement of the F--LW (SEQ ID NO:4) amino acids as they appear in human p53, which compounds bind to human MDM2.

35 Suitable modelling techniques are known in the art. This includes the design of so-called 'mimetics' which

involves the study of the functional interactions of the molecules and the design of compounds which contain functional groups arranged in such a manner that they could reproduce those interactions.

5

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or 10 expensive to synthesise or where it is unsuitable for a particular method of administration, eg peptides are unsuitable active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing 15 is generally used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. 20 Firstly, the particular parts of the compound that are critical and/or important in determining the target property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, eg by substituting each residue 25 in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, eg 30 stereochemistry, bonding, size and/or charge, using data from a range of sources, eg spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between 35 atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional

structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of
5 the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to
10 it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be
15 screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

20 In order to identify compounds which are useful in the above described methods, compounds may be screened for interference of the MDM2/p53 interaction. Suitably screening methods would be based upon observations with regard to compounds which interfere with the binding
25 between peptides which comprise or represent the binding site of an oncogene protein such as MDM2 and p53, based upon the information regarding said binding site given herein. Such methods include immunoassay techniques such as radioimmunoassay (RIA) and enzyme linked
30 immunoabsorbent assay (ELISA) which are well known in the art. Particularly suitable techniques are competitive assay techniques where a peptide or reagent which either is or represents one of the peptides or reagents which either is or represents either p53 or the oncogene
35 protein is exposed to a compound under test and the other one of the peptides or reagents which either is or represents either p53 or the oncogene protein. The

presence of bound complex is then detected. This may be achieved either by labelling of the said one of said peptides or reagent for example using a gold or other visible label, or by administering a labelled antibody or sequence of antibodies, one of which includes a label, in a conventional manner. Suitable antibodies for example for MDM2 and p53 are described herein. Suitably one of the peptides or reagents representing p53 or the oncogene protein is immobilised on a support.

10

Hence the invention further provides a method of identifying compounds which interfere with the binding of human MDM2 to human p53, said method comprising immobilising a first peptide molecule,

15

adding a compound to be tested and a second peptide molecule; detecting the presence of bound second peptide at the immobilisation site;

20

wherein one of the first peptide or second peptide is MDM2 or an oncogene protein having a p53 binding site analogous to that of MDM2 or a fragment thereof which includes said binding site, and the other is a fragment of human p53 of from five to twenty eight amino acids including the amino acid residues FxxLW (SEQ ID NO:4) or a peptide analogue thereof.

25

A immunoassay for detecting binding is illustrated hereinafter.

30

A biotinylated peptide containing the MDM2 binding site for p53 from amino acids 16-25 (or smaller peptides as described above containing for example TFSDLW) (SEQ ID NO:2) would be immobilised on a streptavidin coated ELISA plate. Recombinant MDM2 protein would be added to these ELISA plates in the presence of absence of test compounds or agents. This would then be incubated for 2 hours at 4°C. Bound MDM2 would be detected by a standard ELISA procedure. The inhibitory or stimulatory effect of these

35

reagents would be determined by reference to control wells in which no such test compounds were included. Further experimental details are as described hereinafter and the binding assay is illustrated in Figure 7.

5

The invention includes quantitative assays. For example there is provided a method of identifying compounds which interfere with the binding of human MDM2 to human p53, said method comprising binding a predetermined quantity of a first peptide which is detectably labelled to a peptide, adding a compound to be tested; and determining the quantity of the first protein which is displaced from or prevented from binding to the second peptide, wherein one of the first peptide or the second peptide is MDM2 or 10 a peptide having a p53 binding site analogous to that of MDM2, and the other is a fragment of human p53 of from 15 six to twenty eight amino acids including the amino acid residues 18-23 in the sequence of human p53 as set out in WO93/20238, or a peptide analogue thereof.

20

Assays which include fragments of p53 including the MDM2 binding site as characterised above, or peptide analogues thereof form a further part of the invention. The assay may be formulated as a kit which also forms part of the 25 invention. A particularly useful form of the assay would be one which was adapted to test levels of oncogene protein in biological samples. Such a kit would comprise a fragment of p53 or a peptide analogue as binding agent thereof together with an antibody which is specific for 30 the oncogene protein such as MDM2. Alternatively antibody(s) able to detect bound complex may be included in the kit.

This could be used in diagnosis to measure the levels of 35 oncogene protein or MDM2 in blood samples in the case of leukaemias or solid carcinomas such as sarcomas and glioblastomas.

Suitably in the above described assay methods, the oncogene protein is human MDM2 and the other protein comprises a fragment of human p53 of from 12 to 28 amino acids including the sequence TFSDLW (SEQ ID NO:2).

5

The methods can be readily adapted to provide a high throughput screen, for example by carrying out the process in a 96-well format. Automated screening techniques can be applied in these circumstances as would be understood in the art. Compounds from various sources 10 can be screened in large numbers. One potential source of compounds are the available synthetic combinatorial peptide libraries.

15

The use of compounds identified by this screening method in the treatment of tumours forms a further aspect of the invention.

20

Methods of treatment of conditions such as cancer and other malignancies are envisaged by the administration of the compounds of the invention.

25

Hence the invention also provides a method for inhibiting the growth of tumour cells which contain a human MDM2 gene amplification which method comprises administering an effective amount of a compound which interferes with the binding between p53 and an MDM2, said compound being selected from the group consisting of a peptide having up to twenty eight amino acids which is able to disrupt or 30 prevent the binding between p53 and MDM2, or a functional peptide analogue thereof.

35

Preferably in the above-described method of treatment, the compound is a peptide of from six to twenty eight amino acids which has a consensus with a region of human p53 and includes the sequence FxxLW (SEQ ID NO:4) for example TFSDLW (SEQ ID NO:2).

Alternatively, the compound used in the method is peptide analogue such as an organic compound which binds to the same site on MDM2 as the sequence TFSDLW (SEQ ID NO:2).

5 For use in these applications, the compounds are suitably applied in the form of compositions with pharmaceutically acceptable carriers. These may be solid or liquid for carriers and the compositions suitable for oral or parenteral application as would be understood in the art.

10 Dosages of the compounds will depend upon the patient, the particular condition and the nature of the specific compound chosen. For example, when the compound is a peptide fragment dosages of from 0.1 to 10 mg/Kg may be effective.

15 It has been suggested (Picksley and Lane. (1993). Bioessays. 15, 10, 689-690) that mdm2 expression is autoregulated in a feedback loop, via the inhibitory effect of MDM2 protein on the transcriptional activity of wild-type p53. Any interference with the binding between p53 and MDM2 in accordance with the present invention, will affect the p53-MDM2 autoregulatory loop. Given p53's role as guardian of the genome, compounds which have such an effect could enhance the activity of other therapeutic agents.

20
30 Hence in a further aspect the invention comprises a pharmaceutical composition comprising synergistic amount of a compound of the invention in combination with another anticancer therapeutic agent.

35 DNA encoding an MDM2-binding, p53 derived peptide, or multiple copies thereof may also be administered to tumour cells as a mode of administering the peptide. Hence the invention provides a method for inhibiting the growth of tumour cells which contain a human MDM2 gene amplification, the method comprising applying to said

tumour cells a DNA molecule which expresses a polypeptide comprising a portion of p53 or a variant thereof, said portion comprising amino acids 18-23 of p53, said polypeptide being capable of binding to human MDM2.

5

The DNA will typically be in an expression construct, such as a retrovirus, DNA virus, or plasmid-vector, which has the DNA elements necessary for expression properly positioned to achieve expression of MDM2-binding peptide.

10

The DNA can be administered *inter alia* encapsulated in liposomes, or in any other form known to the art to achieve efficient uptake by cells.

15

By identifying the binding site so specifically, the applicants have opened up the possibility of making small therapeutic compounds which will target this site specifically. This is advantageous since small molecules are more likely to be able to penetrate into a cell and hence be therapeutically active. Furthermore the diagnostic process can be effected more accurately and using simpler molecules as a result of this discovery.

20
25

The invention will be described further by reference to the accompanying drawings in which: Figures 1A-1B show Western blots of immunoprecipitates of MDM2, p53 and the MDM2-p53 complex obtained from Clone 6 cells.

30

Figures 2A-2B are graphs showing the results of two-site immunoassays to determine the levels of MDM2, p53 and MDM2-p53 complex in Clone 6 cells grown at 32°C for 24 hours (A) or continuously at 37°C (B).

35

Figures 3A-3C are graphs showing the results of binding MDM2 to a peptide library as determined by ELISA assay using monoclonal antibody 4B2. The library of human and mouse p53 had been challenged with insect cell extract alone (SF9) and insect cell extract expressing mouse MDM2

(SF9 Mus MDM2. The results of peptides numbers 3-50 of the N-terminal to mid region of human p53 are shown in 3A, and the remainder of the human p53 amino acid sequence and the N-terminal sequence of mouse p53 in 3B. 5 3C shows the results from a control experiment using certain peptides from B in order to verify the specificity of the detecting antibody 4B2.

Figure 4 identifies the peptide sequences to which MDM2 10 bound, and defines the consensus binding site on human and mouse p53;

Figures 5A-5C show the key residues required for binding MDM2, antibody DO-1 and antibody Bp53-19 respectively;

15 Figure 6 shows the level of binding between MDM2 and a series of peptides based upon the sequence of amino acids 18-23 of p53; and

20 Figure 7 illustrates an immunoassay procedure of the invention.

The first indication of an interaction between MDM2 25 protein and p53 protein emerged from work on a rat cell line, Clone 6, which expressed a temperature sensitive mutant form of mouse p53 (Barak and Oren, 1992; Michalovitz, D., et al. (1990). Cell, 62, 671-680; Momand, J., et al. (1992). Cell, 69, 1237-1245). MDM2 30 was readily observed to form a complex at 32°C with p53 but was just detectable when cells were grown at 37°C

The formation of a p53-MDM2 complex in Clone 6 cells at 32°C and 37°C was re-examined in a quantitative manner. 35 The results confirm previous immunoprecipitation observations that the level of MDM2 at the lower temperature is significantly elevated, approximately 10-

30 fold greater than that at 37°C, at which temperature MDM2 is only just detectable. Consequently, the p53-MDM2 complex is readily observed at 32°C and not at 37°C. The levels of p53 also vary at the two different
5 temperatures. However, the p53 levels are elevated approximately five fold at 37°C as compared with that at 32°C, -the opposite behaviour to that of MDM2.

Accordingly, the difference in the levels of p53 and MDM2 are likely to have alternative explanations. In the case
10 of MDM2 other groups have established that the increase of MDM2 at 32°C is due to increased transcription of MDM2 due to a conformational change in p53 to a presumed transcriptional active form (Barak, Y., et al. (1993). Embo J., 12, 461-468; Wu, X., et al. (1993). Genes Dev.,
15 7, 1126-1132). The same explanation does not apply for p53 even though wild type p53 is required for p53 expression (Deffie, A., et al. G. (1993). Mol. Cell. Biol., 13, 3415-3423), and is probably explained by the increased half life of the mutant conformation of p53 at
20 37°C (Gannon J.V. et al (1991) Nature, 349, 802-806). Data described herein after using both direct observation of the p53-MDM2 complex by ELISA and immunoprecipitation combined with the indirect inference of the loss of the Bp53-19 epitope suggested that nearly all p53 molecules
25 are complexed to excess MDM2 protein in C6 cells at 32°C. This is not consistent with the powerful p53 dependant transcriptional response seen in these cells at this temperature and suggests that either that complexing to MDM2 is unable to completely inactivate p53 in vivo or
30 that small amounts of "free" p53 may be very active. The complex between p53 and MDM2 may be regulated in cells to release functional p53 at the individual cell level perhaps as a cell cycle dependant response.

35 The present invention is based upon the identification of the minimal MDM2 binding site to be TFSD/GLW (SEQ ID NOS:2 and 3). This site is in a location broadly

reported by other groups to be the MDM2 binding domain of p53, specifically aa1-41 and 13-57 (Oliner, J.D., et al. (1993). *Nature*, 362, 857-860), aa1-52 (Chen, J., et al. (1993). *Mol Cell Biol*, 13, 4107-14) and aa1-159 (Brown, D.R., et al. (1993). *Mol. Cell. Biol.*, 13, 6849-57.)

Notably, a construct generated by Oliner and co-workers encompassing aa13-41 of p53 was not sufficient for MDM2 binding in a three hybrid protein system, and differs from our observations. The disparity might be explained by the close proximity of the fusion protein sequence adjacent to the TFSDGLW (SEQ ID NO:7) sequence at aa18-23 as the present data does show that flanking sequences do contribute in a minor way to MDM2 binding. The TFSD/GLW (SEQ ID NOS:2 and 3) sequence is very closely adjacent to the transactivation domain aa20-42 (Unger, T., et al. (1992). *Embo J.*, 11, 1383-1390), and as shown by others the binding of MDM2 to this site interferes with the transcriptional activity of p53 (Oliner, J.D., et al. (1993). *Nature*, 362, 857-860). While substitution analysis of the MDM2 binding site on p53 identified the TFSD/GLW (SEQ ID NOS:2 and 3) sequence to be the key region required for MDM2 to bind p53, other residues flanking this site also contribute in a minor way to MDM2 binding, but clearly the TFSD/GLW (SEQ ID NOS: 2 and 3) sequence is a minimal target for agents that might disrupt complex formation without effecting the transactivation activity (for which as yet the key residues are undetermined). The first two residues TF are part of the conserved box I, and the latter four SD/GLW (SEQ ID NOS:8 and 9) are outside but are also part of a region of p53 that is conserved from Xenopus to man.

The corresponding binding site on MDM2 for p53 has variously been reported to be between aa1-121, 19-102 (Chen, J., et al. (1993). *Mol Cell Biol*, 13, 4107-14) together with aa102-294 or 249-491, and also 1-221

(Brown, D.R., et al. (1993). Mol. Cell. Biol., 13, 6849-57). Notably, a monoclonal antibody against the N-terminal region of human MDM2, 3G5 (maps at aa59-89) is able to immunoprecipitate MDM2 but not co-
5 immunoprecipitate p53 (Chen, J., et al. (1993). Mol Cell Biol, 13, 4107-14), an analogous observation to our findings with antibody Bp53-19.

The binding of MDM2 to p53 peptides has obvious parallels
10 to a similar study that used small peptides to identify the binding sites of Adenovirus E1A and human papilloma virus E7 for a range of proteins including retinoblastoma protein, p107, cyclin A and p130 (Dyson, N., et al. (1992a). J. Virol., 66, 4606-4611). The MDM2 binding site on p53, appears to be a single domain rather than
15 two domains as in the case of E1A and E7. The MDM2 binding site on p53 overlaps precisely with a highly immunogenic epitope on the protein; many independently isolated monoclonal antibodies to p53 recognise the site,
20 and antibodies to it are present in the sera of cancer patients (Schlichtholtz, B., et al. (1993). Cancer Res., 52, 6380-6384). This suggests that it has an exposed and defined structure. It is possible that the amino acid sequence of the complementarity determining regions of
25 these antibodies will show homology to the p53 binding site of MDM2. It also suggests that anti-p53 antibodies used to examine p53 levels where high levels of MDM2 are present must be chosen with care. Binding of MDM2 to this site may be regulated by phosphorylation since there
30 is a DNA-dependent kinase site at serine 20 (Less-Miller, S.P. et al. (1990). Mol. Cell. Biol., 10, 6472-6481) and other phosphorylation sites at serine 6, 9 and 15 (Samad, A., et al. (1986). Proc. Natl. Acad. Sci. U.S.A., 83, 897-901; Meek, D.W. et al. (1988). Mol. Cell. Biol., 8, 35 461-465; Meek and Eckhardt, 1988).

The following examples are provided to exemplify various

aspects of the invention and are not intended to limit the scope of the invention.

5 In these examples, the following materials and methods were used.

Materials and methods

Cell culture

10 Clone 6 cells (Michalovitz et al., 1990) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS at either 32 or 37°C. The *Spodoptera frugiperda* cell line, SF9, was grown at 27°C in ExCell 400 medium (J.R. H. Biosciences, Sera-Lab, UK) supplemented with 5% FCS and glutamine.

15

Expression of MDM2 in insect cells

20 The mouse *mdm2* gene was obtained from a mouse prostate cell line (Lu et al., 1992) by polymerase chain reaction and then cloned into a *Spodoptera frugiperda* expression vector pVL1393 using standard DNA and baculovirus expression techniques. An expression clone was identified by the production of a 90-95kDa protein that was recognized by anti-MDM2 antibodies.

25

Antibodies

p53 protein was detected using the polyclonal sera CM1 (Midgley, C.A., et al. (1992). J. Cell. Sci., 101, 183-189), or monoclonal antibodies PAb421 (Harlow E et al., 1981) J. Virol., 39, 861-869) and Bp53-19 (Bartek J., et al (1993). J. Pathol., 169, 27-34). MDM2 was detected using rabbit anti-MDM2 polyclonal sera (Barak, Y., et al. (1993). Embo J., 12, 461-468) or monoclonal antibody 4B2 (Chen et al., 1993) and SMP14 (a previously unreported monoclonal antibody raised by us against a peptide, CSRPSTSSRRRAISE, (SEQ ID NO:10) containing part of the human MDM2 sequence from aa154 to 167 (Oliner, J.D., et al. (1992). Nature, 358, 80-83) the first cysteine is not

part of the MDM2 sequence but was added to provide an extra coupling option). An antibody, PAb419, raised against SV40 large T antigen (Harlow, E., et al. (1981). J. Virol., 39, 861-869) was used as an irrelevant control
5 for immunoprecipitations.

Immunoprecipitation

Cells were lysed in ice-cold NET buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 5mM EDTA, 1% NP40) containing 1mM phenylmethylsulphonyl fluoride, for 30 min at 4°C.
10 Debris was removed from the cell extract by centrifugation at 14,000 rpm in a refrigerated Eppendorf centrifuge. The immunoprecipitation procedure was essentially as previously described (Gannon, J.V., et al.
15 (1990). Embo J., 9, 1595-1602) using 1 μ g of purified mouse monoclonal antibody, and Protein G Sepharose beads (Pharmacia) for both pre-absorption of the cell extracts and subsequent isolation of the antibody-protein complex.

20 Screening of p53 peptide library

Peptide libraries of the entire human p53 protein and a partial N-terminal region of the mouse p53 protein was obtained from Chiron Mimotopes P/L (Victoria, Australia).
25 The libraries were in the form of 15 mer peptides linked to biotin via an additional peptide spacer region of serine-glycine-serine-glycine, and each peptide shared a 5 amino acid overlap with the previous peptide in the primary sequence. ELISA plates were coated with 100 μ l of 5 μ g/ml streptavidin (Vector labs) per well and incubated overnight at 37°C and then blocked with phosphate buffered saline (PBS) containing 2% bovine serum albumin (BSA) for 1 hour at room temperature. The stock biotinylated peptides were diluted to 5 μ g/ml in PBS
30 containing 0.1% BSA and 50 μ l of each were plated into designed wells and then incubated at room temperature for 1 hour. The plates were washed four times with PBS
35

containing 0.1% Tween 20 before addition of the cell
extract (50 µl of 1-4 mg/ml per well) or purified
protein. The plates were incubated at 4°C for 2-3 hours,
before washing four times with PBS containing 0.1% Tween
5 20 to remove unbound protein. In the case of cell
extracts bound protein was detected with the appropriate
primary antibody at 1-3 µg/ml, and followed by an anti-
mouse horse radish peroxidase conjugate and 3'3'4'4'-
10 tetramethyl benzidine (TMB) substrate as in the standard
ELISA assay (Harlow, E., et al (1988). Antibodies: a
laboratory manual. New York. Cold Spring Harbor
Laboratory Press and Lane, 1988).

The levels of p53, MDM2, and complexes thereof were
15 determined by a two site immunoassay using stated
antibodies. Mouse monoclonal antibodies were used as the
solid phase by incubating Falcon microtitre dish wells
with 50 µl of a 30 µg/ml solution of purified antibody
overnight at 4°C. The plates were blocked with 2% bovine
20 serum albumin in PBS for 2 h at room temperature, and
washed with PBS. Cell extracts were prepared as
described for immunoprecipitations and then serially two-
fold diluted before adding 50µl per well and incubating
at 4°C for two hours. The plates were then washed with
25 0.1% NP-40 in PBS, before addition of 50 µl of detecting
polyclonal antisera at 1/1000 dilution. The plates were
washed again with 0.1% NP-40 in PBS and 50 µl of 1/1000
dilution peroxidase conjugated swine anti-rabbit Ig serum
(DAKO) was added for 2 h, then visualised by the TMB
30 reaction.

EXAMPLES

Example 1

Immunoprecipitation of MDM2, p53 and the MDM2-p53 complex
35 The observation that the rat cell line, Clone 6 expressed
a temperature sensitive mutant form of mouse p53 was
reexamined using a panel of p53 monoclonal antibodies.

Western blots were obtained of immunoprecipitates of MDM2, p53 and the MDM2-p53 complex from Clone 6 cells grown at 32°C for 24 hrs (Figure 1A) or continuously at 37°C (Figure 1B). The immunoprecipitates were obtained 5 using 1 μ g of purified antibody which were as follows: in lanes 1 and 4, - PAb421; in lanes 2 and 5, - Bp53-19; and in lanes 3 and 6, - 4B2. MDM2 was detected in lanes 1, 2 and 3 using SMP14 antibody supernatant and rabbit anti-mouse horse radish peroxidase conjugate; and p53 detected 10 in lanes 4, 5 and 6 using a 1 in 200 dilution of DM-1 and swine anti-rabbit horse radish peroxidase conjugate. An irrelevant antibody, PAb419, did not immunoprecipitate either MDM2 or p53 from cell extracts prepared at either 32°C or 37°C (data not shown). The molecular weight of 15 the markers are given in kDa.

It was surprisingly found that one of the antibodies, Bp53-19, failed to immunoprecipitate p53 from Clone C6 cells grown at 32°C for 24 hours, but efficiently 20 precipitated p53 from cells grown continuously at 37°C (compare Figure 1A track 5 with Figure 1B track 5), whereas PAb421 precipitated p53 at both temperatures (Figure 1A track 4 and 1B track 4). Investigations were then carried out to determine whether Bp53-19 would co- 25 immunoprecipitate MDM2 with p53. From the immunoprecipitation western data in figure 1A and 1B it is clear that Bp53-19 does not co-immunoprecipitate MDM2 from cell extracts grown at 32 or 37°C (track 2 in figure 1A and B). Other p53 antibodies such as PAb421 do 30 however co-immunoprecipitate MDM2 with p53 at 32°C but not at 37°C (track 1 figure 1A and B). Conversely, antibodies against MDM2 such as 4B2, Figure 1, and SMP14 (data not shown) co-immunoprecipitate p53 at 32°C but not at 37°C (track 6 Figure 1A and B). The two bands 35 recognized by 4B2 (and SMP14) at just below 80 kDa are truncated forms of rat MDM2, as full length migrates on an SDS-PAGE gel with an apparent relative molecular mass

of 90kDa, multiple forms of MDM2 are often observed (Chen, J., et al. (1993). Mol Cell Biol, 13, 4107-14).

Example 2

5 Two-site immunoassay to determine levels of MDM2, p53 and MDM2-p53 complex

Two-site immunoassays were carried out to determine the levels of MDM2, p53 and MDM2-p53 complex in Clone 6 cells grown at 32°C for 24 hrs (Figure 2A) or continuously at 10 37°C (Figure 2B). In Figure 2A the coating antibodies were one of the following purified antibodies as stated in the figure legends: 4B2, 421 and Bp53-19, probed with rabbit anti-p53 serum CM1 or rabbit anti-MDM2 serum, and then detected using swine anti-rabbit horse radish 15 peroxidase conjugate and TMB as substrate. At 37°C the MDM2-p53 complex was undetectable by any combination of antibodies.

20 The two-site immunoassays of the levels of MDM2, p53 and MDM2-p53 complex at 32°C and 37°C are consistent with the immunoprecipitation results of Example 1. A striking feature apparent from the data in figure 2A is that the levels of p53 and p53-MDM2 complex are very similar suggesting that most, but not all, p53 is in complex with 25 MDM2 at 32°C. The inability of Bp53-19 to detect a p53-MDM2 complex at 32°C is again notable since other combinations of antibodies are able to do so.

30 From comparison of the two-site immunoassays at 32°C and 37°C it is clear why MDM2 is not immunoprecipitated at 37°C, as the levels of MDM2 protein are very much lower and are only just detectable. No MDM2-p53 complex could be detected by the two-site immunoassay of cell extracts prepared at 37°C, see figure 2B, where the data for the 35 4B2 (as the capture antibody) and CM1 (as the detecting antibody) combination of antibodies is shown (similarly antibodies PAb421 or Bp53-19 and rabbit anti-MDM2

polyclonal did not detect the complex). The diminished level of MDM2 at 37°C, less than 10% of that at 32°C, is in contrast to the situation with p53 which is elevated approximately 5 fold relative to the levels at 32°C.

5

The explanation for the ability of PAb421 and 4B2 only being able to coprecipitate p53 and MDM2 together at 32°C, but not at 37°C is consistent with difference in levels of MDM2 at the two temperatures, and also with the 10 published observations that *mdm2* expression is dependent on the "wild-type" form of p53 predominantly present at 32°C.

15 The failure of Bp53-19 to co-immunoprecipitate MDM2 or detect the p53-MDM2 complex at 32°C is unexpected for two reasons. Firstly, the two-site assay suggests there is MDM2 protein in excess, which is able to form complexes with p53 as detected by the capturing antibodies PAb421 and 4B2. Secondly, the two-site immunoassay at 37°C 20 suggests that Bp53-19 is almost as efficient as PAb421 at recognizing p53 in the cell extracts. The simplest interpretation for this observation is that Bp53-19 recognizes the same region on p53 that MDM2 binds to.

25

Example 3

Identification of MDM2-p53 binding site

It has previously been shown that Bp53-19 and MDM2 interact with the amino acid terminal end of p53 (Stephen 30 et al manuscript in preparation; Oliner, J.D., et al. (1993). *Nature*, 362, 857-860). A complete peptide library of the human p53 protein, and a partial peptide library of the mouse p53 protein were available to identify the region to which MDM2 binds. The human p53 sequence starts at peptide number 3 and ends at peptide 35 79, and each peptide consists of 15 amino acids, with the last five amino acids being present in the next peptide

along. The mouse p53 sequence is partial and consists of the N-terminal sequence from amino acid 1-92, again each overlapping the next and previous peptide by five amino acids.

5

These libraries consisted of 15 amino acid long sections of the p53 primary amino acid sequence, that consecutively overlapped by 5 amino acids, and were each attached to biotin via a 4 amino acid long spacer. By immobilizing the biotinylated peptides on streptavidin coated ELISA plates the MDM2 binding site on p53 could be quickly identified if it was encompassed within a stretch of fifteen amino acids or less. Extract containing MDM2 was added to an ELISA plate with the peptide library bound to it, and the bound MDM2 protein was later detected using monoclonal antibody 4B2 and the standard ELISA assay. Several sources of recombinant MDM2 protein were used to challenge the p53 library, these included crude extracts and partially purified preparations of human and mouse MDM2 expressed in E.coli and also mouse MDM2 expressed in insect cells; - all forms identified the same peptides in the p53 library. The results using the mouse MDM2 expressed in insect cells are shown in figure 3A and B. The peptide library was challenged with insect cell extract alone, SF9, and insect cell extract expressing mouse MDM2, SF9 Mus MDM2. Binding of MDM2 to the peptides was determined by an ELISA assay using monoclonal antibody 4B2, and then detecting bound antibody with rabbit anti-mouse Ig conjugated horse radish peroxidase and TMB substrate. In Figure 3C is shown the results from a control experiment using peptides 59, 71, 83 and 95, as used in Figure 3B but conducted in the presence or absence of extract to verify the specificity of the detecting antibody, 4B2. The results are presented alongside the ELISA readings for extract of insect cells alone not expressing mouse MDM2. The specificity is remarkable, - suggesting a strong

interaction between MDM2 and p53 derived peptides. From the controls shown in figure 3C it can be seen that the binding is only observed in the presence of extract expressing MDM2, and is not due to the antibody recognizing the peptide alone, moreover identical results were obtained using SMP14 as the primary detecting antibody (data not shown).

The four peptides that bind MDM2 are shown in figure 4.
10 Peptides 5 and 6 identify a site at the N-terminal end of human p53, whereas peptides 83 and 84 identify the corresponding region in the N-terminal end of mouse p53. Collectively, these four peptides define the consensus MDM2 binding site on p53 to be -QETFSD/GLWKL-, (SEQ ID NOS:11 and 12) the aspartate to glycine being the only
15 amino acid difference between the human and mouse sequence. The peptides involved in binding MDM2 are also those recognized by the p53 antibodies DO-1 and Bp53-19 (Stephen et al, manuscript in preparation).

20 To define key residues on p53 that are involved in the interaction with MDM2 a form of the consensus binding site sequence -QETFSDLWKL- (SEQ ID NO:11) was modified by substituting alanine at each position in the sequence and determining what effect this had on the binding of MDM2 from the insect cell extract expressing MDM2. This experiment was conducted in concert with examining the effect on binding of the antibodies DO-1 and Bp53-19. The results are presented in figure 5. The amino acid sequences are as stated. In A the sequence
25 QETFSDLWKLPPENN (SEQ ID NO:1) represents the sequence of peptide 6 from figure 3 and SPDDIEQWFTEDPGP (SEQ ID NO:13) is an irrelevant peptide control. Formally the first serine residue on the stated peptide is part of the spacer coupling the consensus peptide to biotin, since serin also precedes the consensus p53 sequence this residue was also substituted with alanine. With regard
30
35

to MDM2 binding all alanine substitutions in the consensus binding site reduce the level of binding as measured by ELISA, however, the key residues would appear to be TFSDLW (SEQ ID NO:2) as substitutions in these positions reduce the amount of MDM2 binding to less than 15% of that seen with the unchanged consensus sequence. Interestingly, a higher level of binding of MDM2 is observed to the smaller consensus peptide rather than to peptide 6 (QETFSDLWKLLPENN) (SEQ ID NO:1) of the p53 peptide library reaffirming the definition of the binding site. In the case of monoclonal antibody DO-1 binding to the consensus sequence the key residues are ETFSDLK, (SEQ ID NO:14) with D and K being the most crucial. The importance of the aspartate residue to the DO-1 epitope is consistent with the report that DO-1 only recognizes human p53 and not mouse p53, - the only difference being an aspartate to glycine change. While this difference has a critical affect on DO-1 binding it does not grossly affect the interaction of MDM2 Δ with the protein or peptides. However substitution of alanine for aspartate at this position blocks binding of all three protein ligands. The ability of MDM2 to distinguish alanine from either glycine or aspartate at this position may imply that the polar environment of this region of the binding site is critical for the interaction. It has also been established from phage display libraries that the epitope of DO-1 is FSDLWKL (SEQ ID NO:15) (Stephen et al, manuscript in preparation), which is in agreement with our observations on key residues. For the antibody Bp53-19 the alanine substitution series identifies the key residues to be F-DLW- (SEQ ID NO:16) with the latter three residues being the most crucial, and is similar to the requirements for MDM2 binding to the consensus binding site. Not surprisingly, it was found that the pre-binding of antibody Bp53-19 onto he SQETFSDLWKL (SEQ ID NO:17) biotinylated peptide blocked binding of MDM2 to the peptide when added later (data not shown).

Example 4

Further characterisation of the p53-MDM2 binding site

The process of Example 3 was repeated but using insect cells infected with baculovirus expressing Mus MDM2 from SF9 cells in two 180ml² tissue culture flasks. The extract was prepared in approximately three mils of lysisi buffer to give a concentrated supernatant of 13 mg/ml.

As before, the MDM2 binding site was defined by alanine substitution of the p53 derived peptide, SQETFSDLWL (SEQ ID NO:18) and the results are shown in Figure 6.

Additionally other conserved substitutions were tested (i.e those commonly seen in highly conserved proteins of identical function) and the results are also shown in Figure 6. With a higher protein concentration, (13 mg/ml as opposed to 1-4 mg/ml) the alanine substitution experiment reveals the same six amino acids are important (¹⁸TFSDLW²³) (SEQ ID NO:2) in addition to glutamate (ETFSDLW) (SEQ ID NO:19). This data however establishes that the most critical residues are F--LW (SEQ ID NO:4) as these are intolerant of both alanine substitutions and some, if not all of the conserved substitutions.

A further interesting observation relates to the fact that substitution of the aspartate residue for the glutamate enhances binding of MDM2, indicating that such a residue may usefully be included in the therapeutic peptides of the invention. This also illustrates that this approach can lead to the discovery of agents that have enhanced binding to MDM2.

Additional References

Dyson, N., et al. (1992b). J. Virol., 66, 6893-6902.

Houghten, R.A., et al. (1991). Nature, 354, 84-86.

5 Lu, X., et al (1992). Cell, 70, 153-161.

CLAIMS

1. A compound for use in interfering with the binding between p53 and MDM2 or a protein having a p53 binding site analogous to that of MDM2, which compound comprises a peptide having up to twenty eight amino acids which is able to disrupt or prevent binding between p53 and MDM2, or a functional peptide analogue thereof.
5
- 10 2. The compound of claim 1 which comprises a peptide having from 4 to 10 amino acids.
- 15 3. The compound of claim 1 or claim 2 which comprises a peptide which comprises a fragment of p53 protein which includes at least some of amino acids 18-23 within the sequence of human p53, or a peptide analogue thereof.
- 20 4. The compound any one of the preceding claims which comprise a peptide comprising the sequence FxxLW (SEQ ID NO:4).
- 25 5. The compound of claim 4 wherein the peptide comprises the sequence FxELW (SEQ ID NO:5).
6. The compound of claim 4 or claim 5 wherein the peptide comprises the sequence TFSDLW (SEQ ID NO:2).
- 30 7. The compound of any one of the preceding claim wherein the oncogene protein is human MDM2.
8. The compound of claim 1 which comprises the compound an organic compound which is modelled to resemble the three dimensional structure of the amino acid residues F-LW (SEQ ID NO:4) as they appear at amino acid residues 19-23 in human p53 and which binds to human MDM2.
35
9. A method of identifying compounds which interfere

with the binding of oncogene protein to p53 which comprises exposing a fragment of p53 protein which includes at least some of amino acids 18-23 within the sequence of human p53, or a peptide analogue thereof, to the oncogene protein or a binding fragment thereof in the presence of a compound under test, and detecting bound complex of either the fragment of p53 or peptide analogue, and/or the compound under test with the oncogene protein or binding fragment.

10

10. A method of identifying compounds which interfere with the binding of human MDM2 to human p53, said method comprising

15

immobilising a first peptide molecule, adding a compound to be tested and a second peptide molecule; detecting the presence of bound second peptide at the immobilisation site;

20

wherein one of the first peptide or second peptide is MDM2 or an oncogene protein having a p53 binding site analogous to that of MDM2 or a fragment thereof which includes said binding site, and the other is a fragment of human p53 of from five to twenty eight amino acids including the amino acid residues FxxLW (SEQ ID NO:4) or a peptide analogue thereof.

25

11. The method of claim 9 or claim 10 wherein the said fragment of p53 or peptide comprises a fragment of human p53 of from 12 to 28 amino acids including the sequence FxxLW (SEQ ID NO:4).

30

12. The method of any one of claims 9 to 11 wherein acid peptide analogue is a peptide which includes sequence FxxLW (SEQ ID NO:4).

35

13. The method of any one of claims 9 to 12 which is adapted to provide a high throughput screen.

14. The method of any one of claims 9 to 13 wherein the compounds are obtained from synthetic combinatorial peptide libraries.
- 5 15. The method of any one of claims 9 to 14 wherein the said oncogene protein is human MDM2.
- 10 16. A method of identifying compounds which interfere comprising binding a predetermined quantity of a first peptide which is detectably labelled to a peptide, adding a compound to be tested; and determining the quantity of the first protein which is displaced from or prevented from binding to the second peptide, wherein one of the 15 first peptide or the second peptide is MDM2 or a peptide having a p53 binding site analogous to that of MDM2, and the other is a fragment of human p53 of from six to twenty eight amino acids including the amino acid residues 18-23 in the sequence of human p53 as set out in 20 WO93/20238, or a peptide analogue thereof.
17. A compound for use in interfering with the binding of p53 and MDM2 a method of claim 1 which is identified by a method according to any one of claims 9 to 16.
- 25 18. A novel compound identified by the method of any one of claims 9 to 18.
- 30 19. A novel compound selected from the group comprising a peptide of from four to twenty eight amino acids which is able to disrupt or prevent the binding between p53 and an MDM2, or a peptide analogue thereof.
- 35 20. The compound of claim 19 which comprises a polypeptide of from six to ten amino acids in length.
21. A polypeptide which interferes with the binding of

human MDM2 to human p53, which compound comprises a polypeptide of from five to twenty eight amino acids in length, said polypeptide being coincidental with or a variant of a portion of p53 protein, said portion including amino acids F--LW (SEQ ID NO:4) as contained within amino acids 18-23 within the sequence of human p53 as set out in WO93/20238.

22. A polypeptide which binds with human MDM2, which compound comprises a polypeptide of from five to twenty eight amino acids in length, said polypeptide comprising a portion of p53 protein, said portion including the amino acid sequence TFSDLW (SEQ ID NO:2).

23. A compound which is modelled to resemble the three dimensional structure of the sequence F--LW (SEQ ID NO:4) as it appears in human p53 and which binds to human MDM2.

24. A compound for use in inhibiting the growth of tumour cells which contain a human MDM2 gene amplification, which compound interferes with the binding between p53 and an MDM2 and comprises a peptide having up to twenty eight amino acids which is able to disrupt or prevent the binding between p53 and MDM2, or a functional peptide analogue thereof.

25. A DNA molecule for use in inhibiting the growth of tumour cells which contain a human MDM2 gene amplification, said DNA molecule encoding a polypeptide comprising a portion of p53 or a variant thereof, said portion comprising amino acids 18-23 of p53, said polypeptide being capable of binding to human MDM2.

26. A pharmaceutical composition comprising an effective amount of a compound selected from the group consisting of a peptide having up to twenty eight amino acids which

is able to disrupt or prevent binding between p53 and MDM2 or a peptide analogue thereof in combination with pharmaceutically acceptable carrier or diluent.

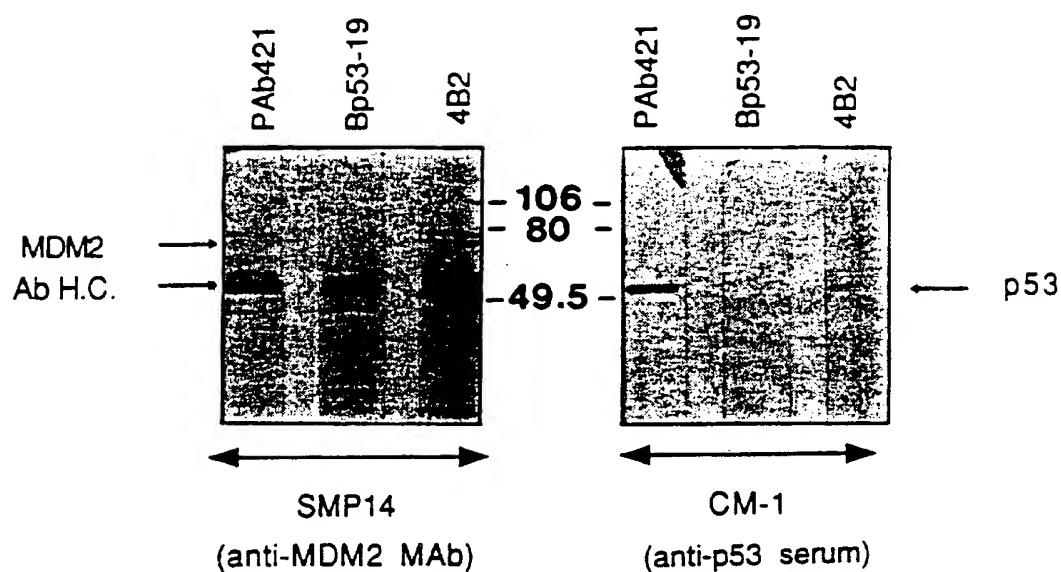
5 27. The composition of claim 21 which further comprises a different anti-tumour chemotherapeutic agent.

10 28. A diagnostic method for detecting the presence of MDM2 comprising applying to a sample under test a fragment of p53 protein which includes at least some of amino acids 18-23 within the sequence of human p53, or a peptide analogue thereof, and detecting the presence of bound complex using antibodies specific for MDM2.

15 29. An assay kit comprising a fragment of p53 protein which includes at least some of amino acids 18-23 within the sequence of human p53, or a peptide analogue thereof, and at least one antibody capable of detecting the presence of bound MDM2.

Fig.1A.

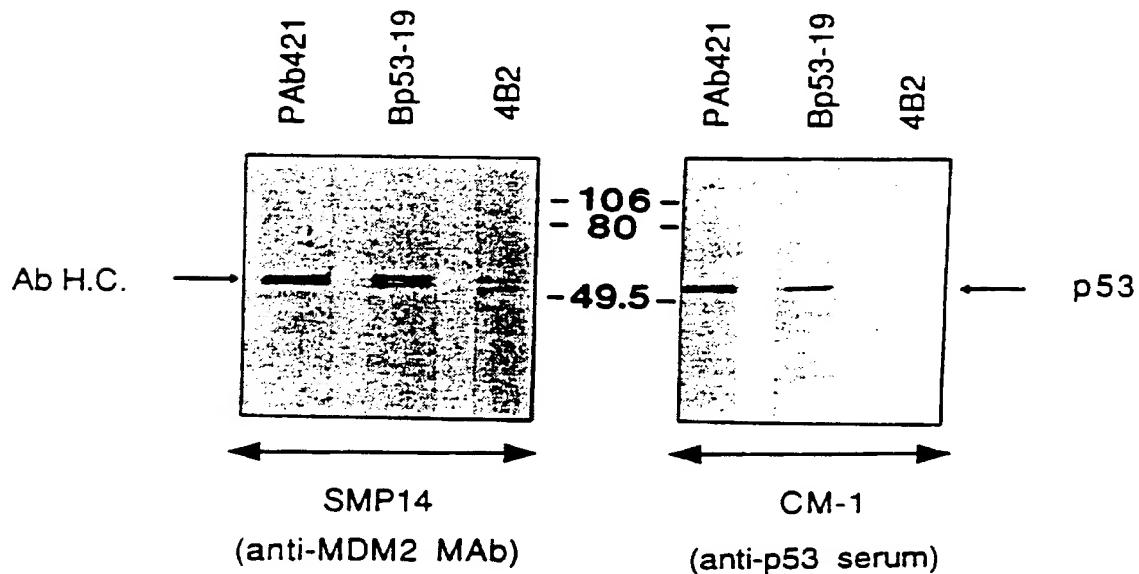
ANTIBODY USED FOR IMMUNOPRECIPITATION



ANTIBODY/SERUM USED FOR DETECTION

Fig. 1B.

ANTIBODY USED FOR IMMUNOPRECIPITATION



ANTIBODY/SERUM USED FOR DETECTION

Fig.2A.

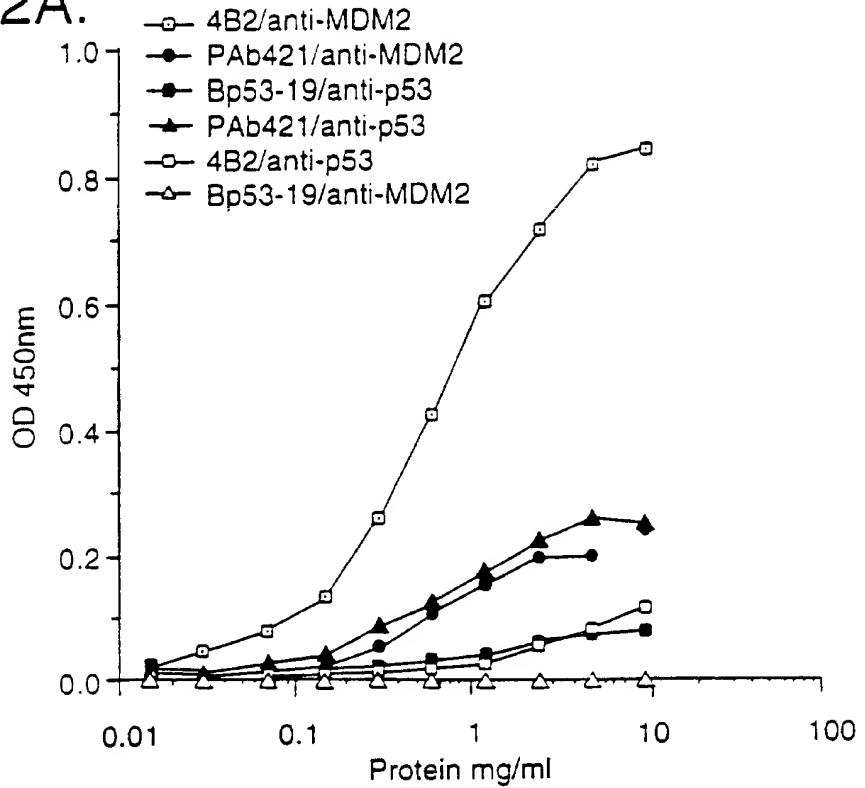


Fig.2B.

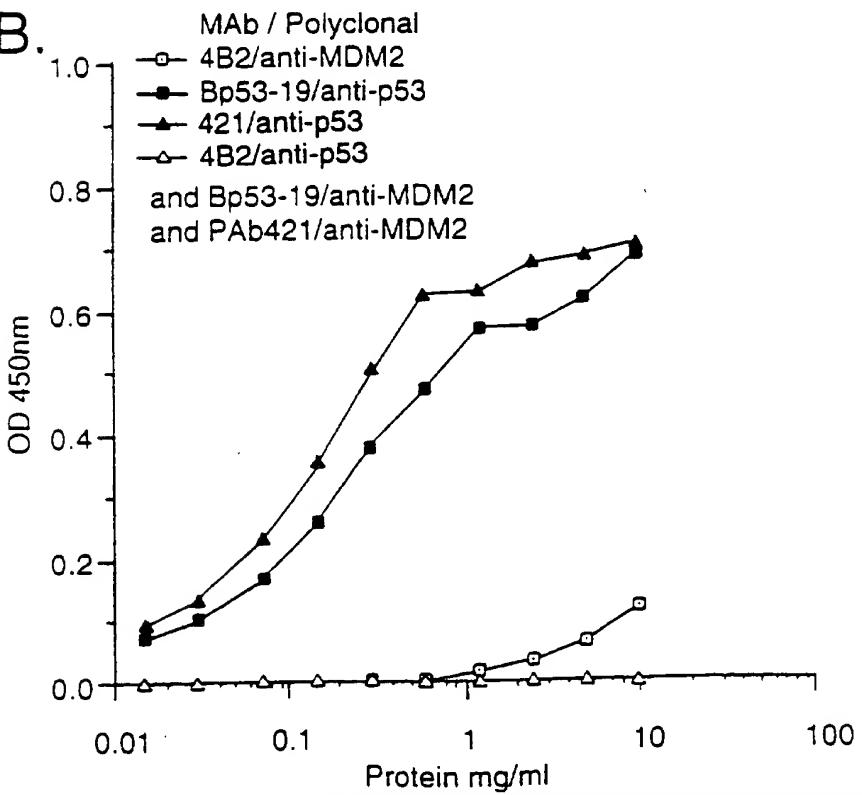


Fig.3A.

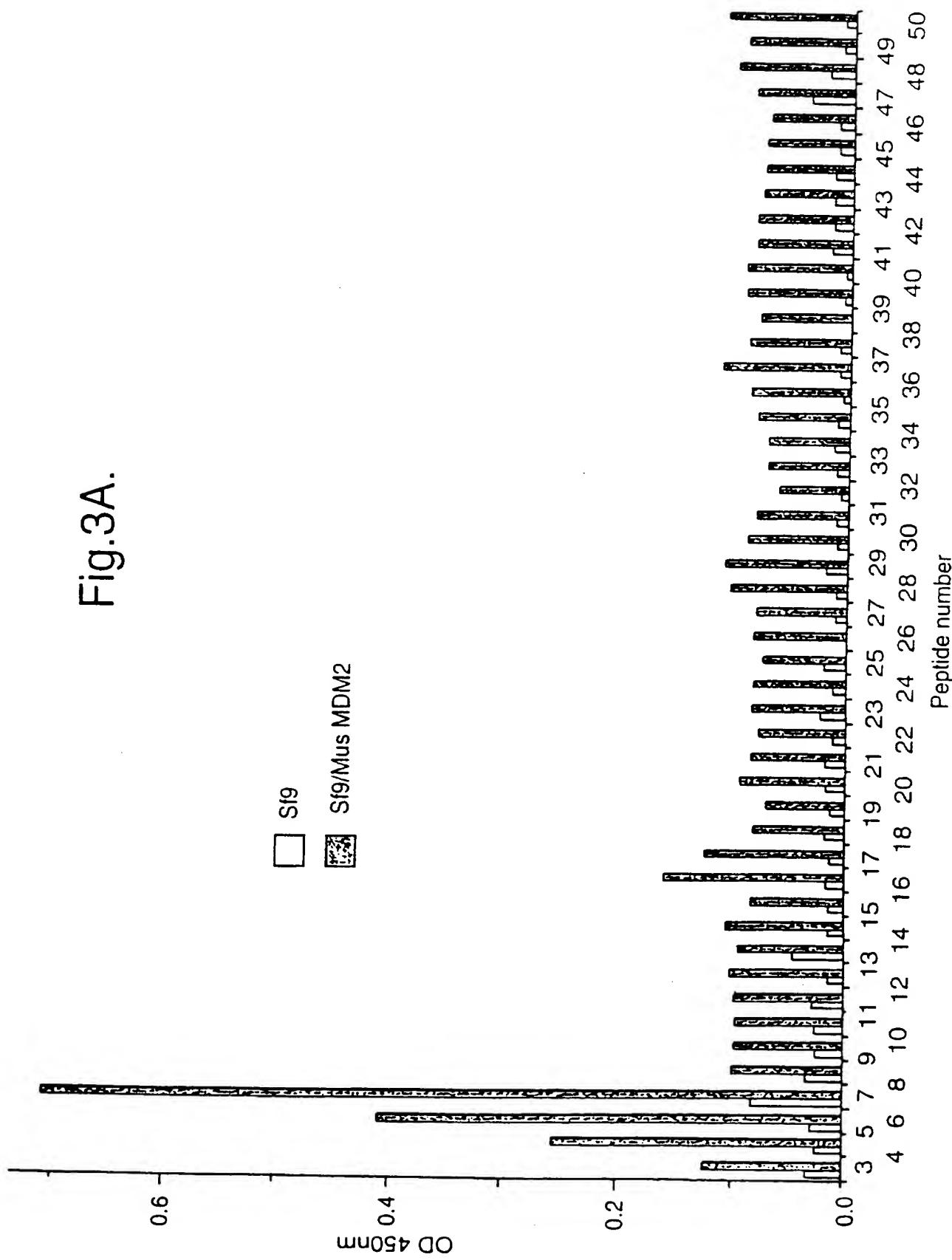
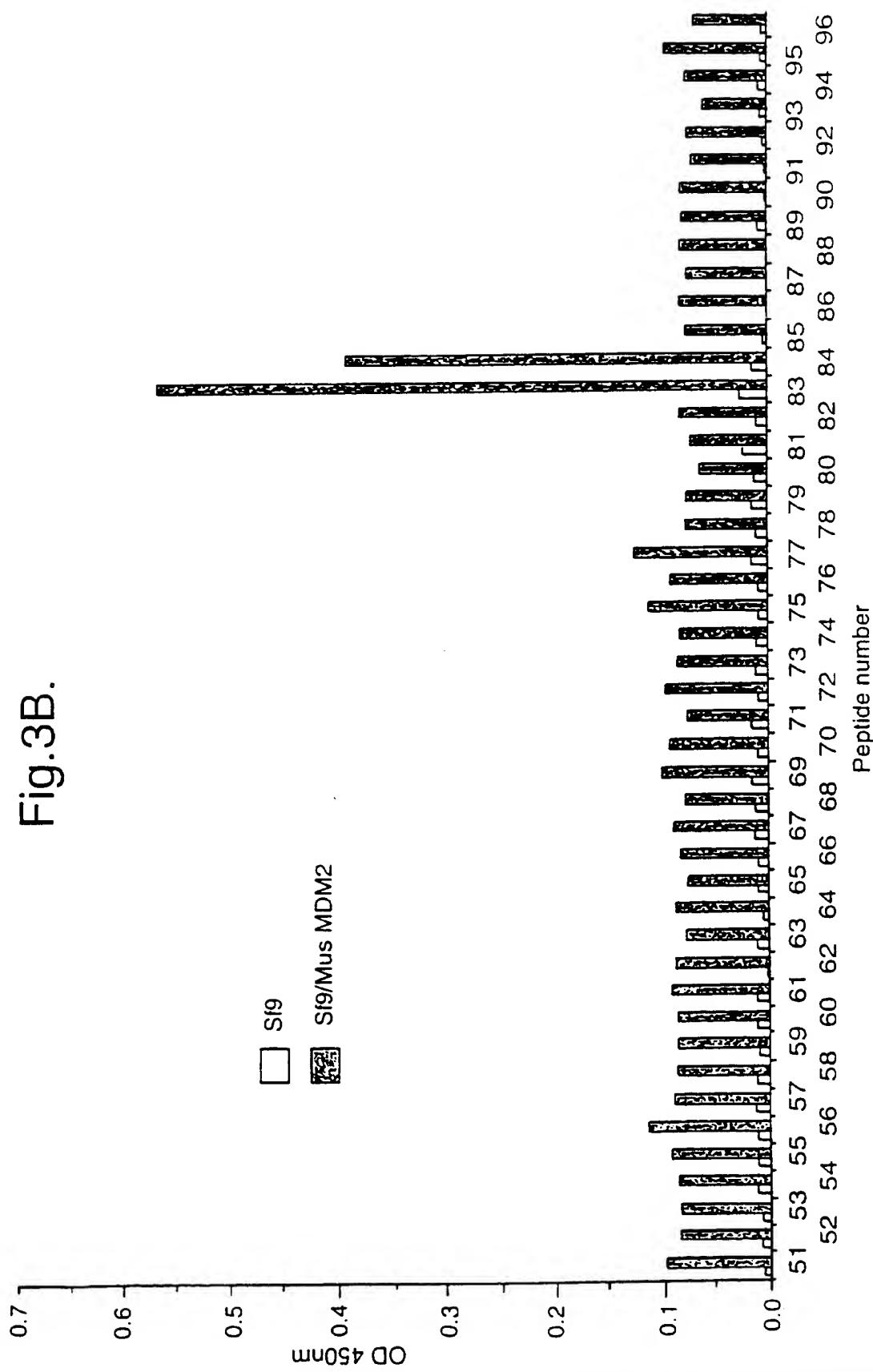


Fig. 3B.



6/9

Fig.3C.

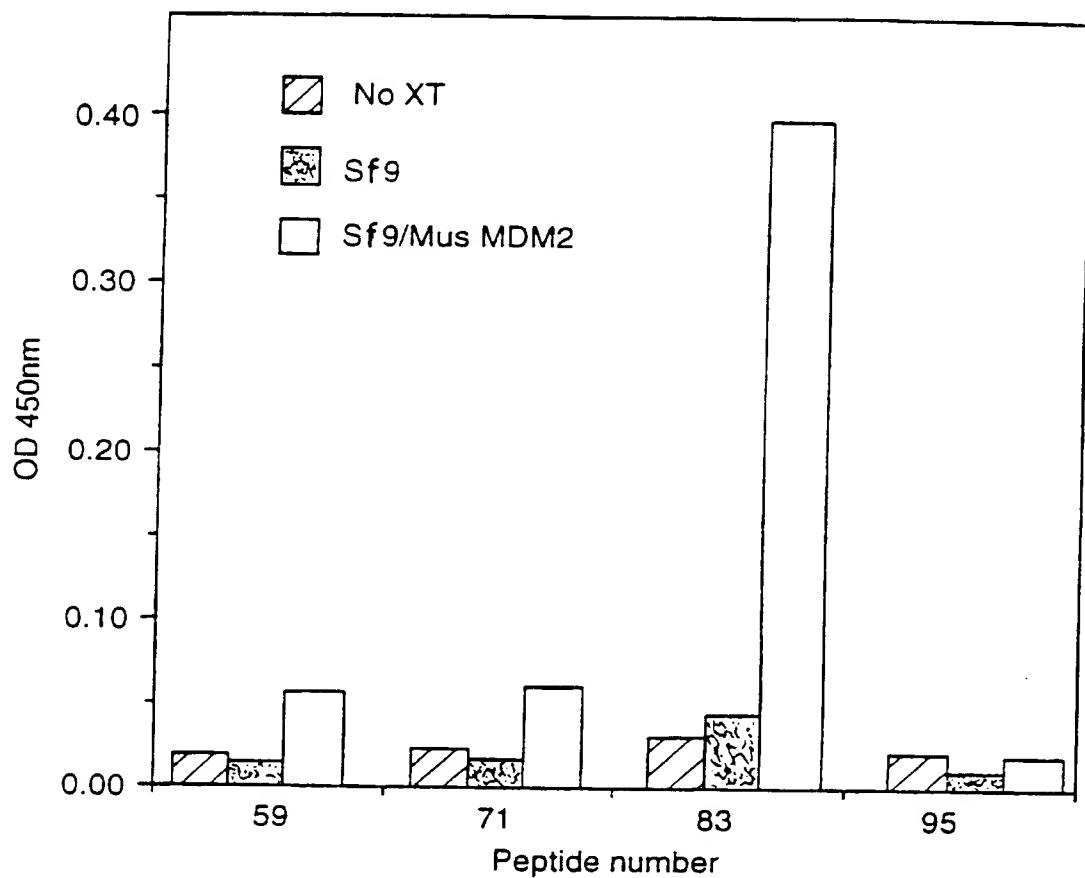


Fig.4.

#5 11 25
 -EPPLSQETFSDLWKL-

#6 16 30
 -QETFSDLWKLPPENN-

#83 13 27
 -PLSQETFSGLWKLLP-

#84 18 32
 -TFSGLWKLLPPPEDIL-

HUMAN p53 SEQUENCE

CONSENSUS MDM2
 BINDING SITE -QETFSD/GLWKL-

MOUSE p53 SEQUENCE

7/9

OD 450nm

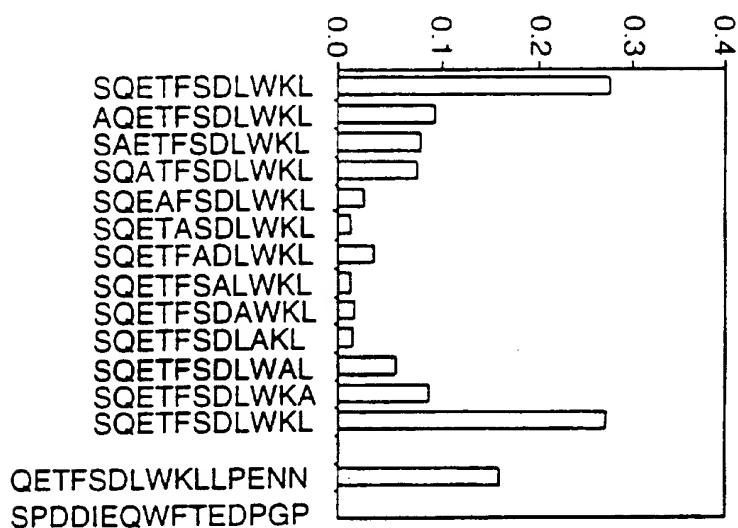


Fig.5A.

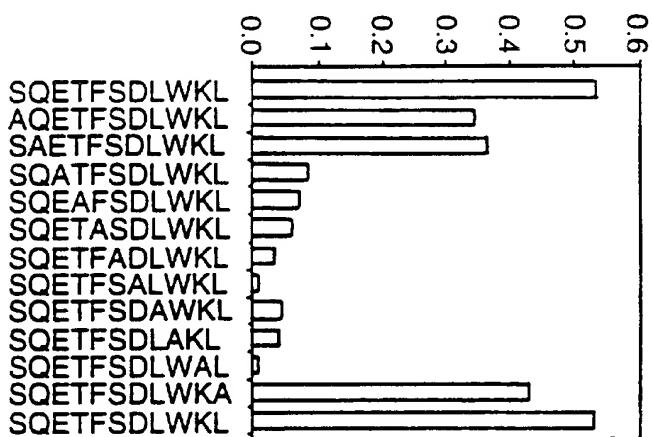


Fig.5B.

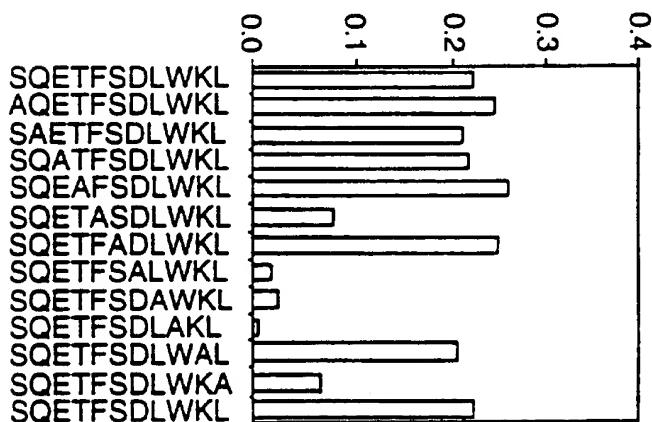
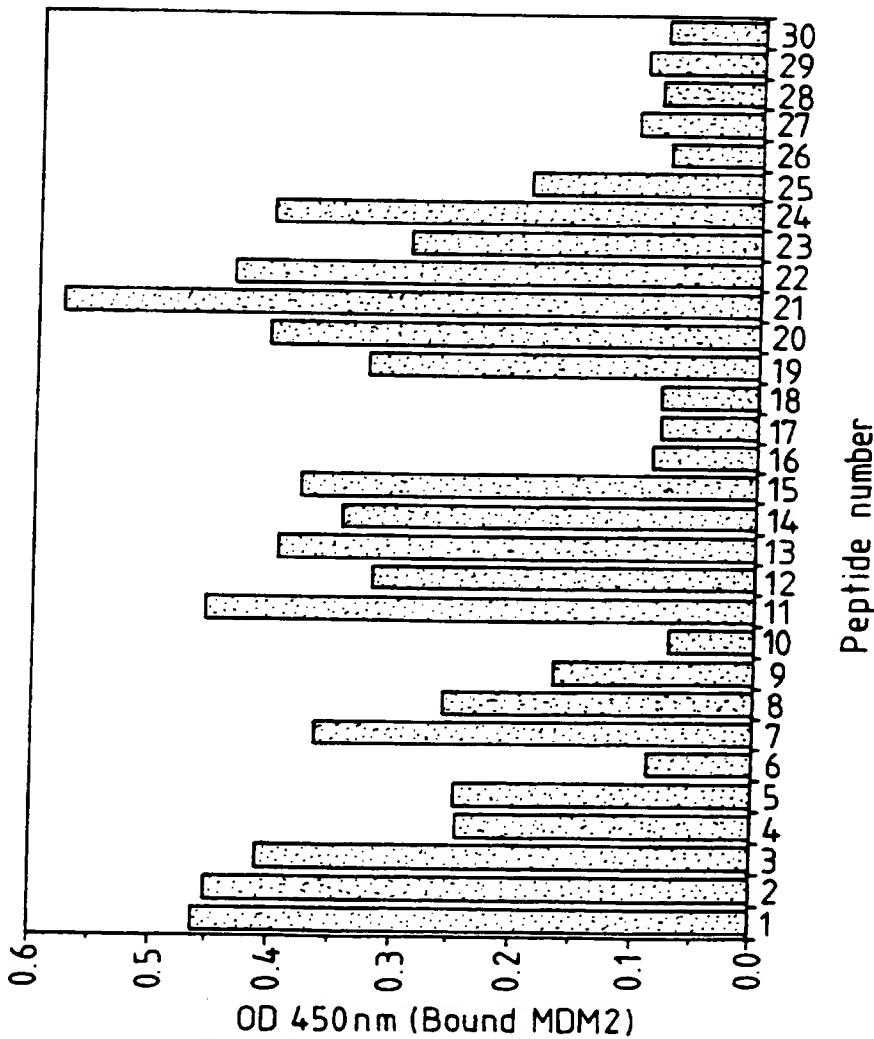


Fig.5C.

Fig.6.
Further information on the key residues required for MDM2 binding

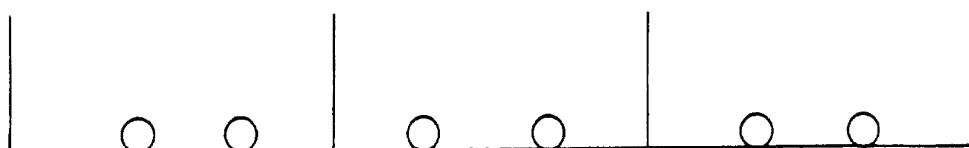
NO.	PEPTIDE	CHANGE
1.	SQETFSDLWKL	-
2.	AQETFSDLWKL	S TO A
3.	SAETFSDLWKL	Q TO A
4.	SQATFSDLWKL	E TO A
5.	SQEAFSDLWKL	T TO A
6.	SQETASDLWKL	F TO A
7.	SQETFADLWKL	S TO A
8.	SQETFSALWKL	D TO A
9.	SQETFSDAWKL	L TO A
10.	SQETFSDLAKL	W TO A
11.	SQETFSDLW <u>AL</u>	K TO A
12.	SQETFSDLW <u>KA</u>	L TO A
13.	DQETFSDLWKL	S TO D
14.	SQETF <u>DD</u> LWKL	S TO D
15.	SQESFSDLWKL	T TO S
16.	SQET <u>I</u> SDLWKL	F TO I
17.	SQET <u>L</u> SDLWKL	F TO L
18.	SQET <u>M</u> SDLWKL	F TO M
19.	SQET <u>T</u> SDLWKL	S TO T
20.	SQETFP <u>D</u> LWKL	S TO P
21.	SQETFE <u>L</u> WKL	D TO E
22.	SQETFS <u>Q</u> LWKL	D TO Q
23.	SQETFS <u>N</u> LWKL	D TO N
24.	SQETFS <u>M</u> WKL	L TO M
25.	SQETFS <u>D</u> IWKL	L TO I
26.	SQETFS <u>R</u> WKL	W TO R
27.	SQETFS <u>E</u> WKL	W TO F
28.	SQETFS <u>Y</u> WKL	W TO Y
29.	SQETFSFLIKL	Tag core seq.
30.	Tag first 15aa	



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Fig.7.

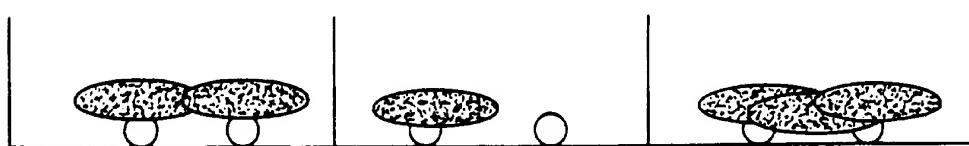
Biotinylated peptide containing MDM2 binding site of p53 added to streptavidin-coated plate



Add MDM2 (peptide or polypeptide) in solution + or - test agents



Wash away unbound MDM2. Determine bound MDM2 by ELISA



ELISA
reading

++
(reference)

+

(below reference)
Test agent disrupts
p53-MDM2 interaction

+++

(above reference)
Test agent enhances
p53-MDM2 interaction

INTERNATIONAL SEARCH REPORT

Inten. Application No.
PCT/GB 95/01719

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/82 C07K14/47 G01N33/574 A61K38/17
A61K38/04

According to International Patent Classification (IPC) or to both nato: classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields sear...

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 20238 (THE JOHN HOPKINS UNIVERSITY) 14 October 1993 cited in the application see page 11 see page 19 last paragraph see page 24 - page 26; example 7 see figure 6 see claims 30, 33, 41 ---	1-19, 21-26, 28,29
X	MOL. CELL. BIOL., vol. 13, no. 7, July 1993 pages 4107-4114, CHEN ET AL. 'Mapping of the p53 and mdm-2 interaction domains' see page 4112	25
Y	----	1-24, 26-29 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

Date of mailing of the international search report

14 December 1995

05. 01. 96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentstaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

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Gac, G

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01719

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENES DEV., vol. 8, no. 10, 15 May 1994 pages 1235-1246, LIN ET AL. 'Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-KD protein' see the whole document ---	1-24, 26-29
X	WO,A,94 08241 (DUETSCHES KREBSFORSCHUNGSZENTRUM STIFTUNG DES ÖFFENTLICHEN RECHTS) 14 April 1994 see page 3 third paragraph see page 19 fragment 8 seepage 25 fragment 8 ---	1-6, 18-23
X	MOL. CELL. BIOL., vol. 12, no. 11, November 1992 pages 5051-5049, LEES-MILLER ET AL. 'Human DNA-activated protein-kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53' see table 1 ---	1-8, 17-24
P,X	ONCOGENE, vol. 9, no. 9, September 1994 pages 2523-2529, PICKSLEY ET AL. 'Immunochemical analysis of the interaction of p53 with mdm2 ; fine mapping of the mdm2-binding site on p53 using synthetic peptides' see the whole document ---	1-29
X	WO,A,94 10306 (LABORATOIRES EUROBIO) 11 May 1994 see the whole document ---	1-6, 17-23
A	WO,A,94 00601 (THE TRUSTEES OF PRINCETON UNIVERSITY) 6 January 1994 see the whole document -----	1-29

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal application No
PCT/GB 95/01719

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9320238	14-10-93	US-A-	5411860	02-05-95
		AU-B-	4278893	08-11-93
		CA-A-	2133306	14-10-93
		EP-A-	0635068	25-01-95
		JP-T-	7505294	15-06-95
		US-A-	5420263	30-05-95
-----	-----	-----	-----	-----
WO-A-9408241	14-04-94	EP-A-	0614531	14-09-94
		JP-T-	7501711	23-02-95
-----	-----	-----	-----	-----
WO-A-9410306	11-05-94	FR-A-	2698367	27-05-94
		AU-B-	5424694	24-05-94
		CA-A-	2148274	11-05-94
		EP-A-	0666913	16-08-95
-----	-----	-----	-----	-----
WO-A-9400601	06-01-94	AU-B-	4544393	24-01-94
		AU-B-	4545193	24-01-94
		CA-A-	2137822	06-01-94
		EP-A-	0654092	24-05-95
		WO-A-	9400603	06-01-94
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